IZSAM G.CAPORALE TERAMO

La Risonanza Magnetica Nucleare nel Settore Alimentare

Teramo, 11 - 12 Luglio 2013

Rintracciabilità di alimenti mediante NMR: formaggi e miele

UNIVERSITÀ DEGLI STUDI DI PADOVA

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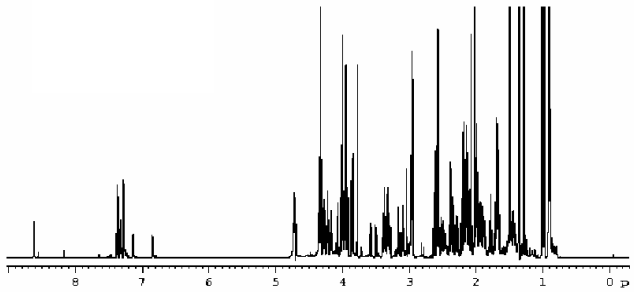
Tracciabilità o Rintracciabilità ???

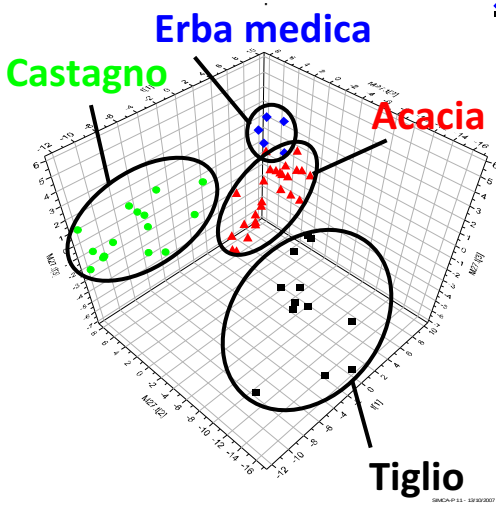
La tracciabilità (track) è l'attività di lasciare una traccia **(per esempio mediante apposita etichettatura)**

La rintracciabilità (trace) è invece la capacità di ritrovare la traccia

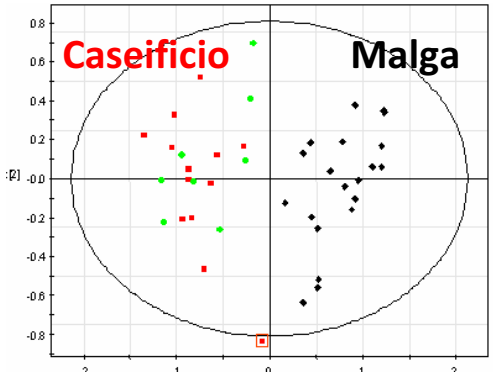
Spettro NMR di matrici alimentari



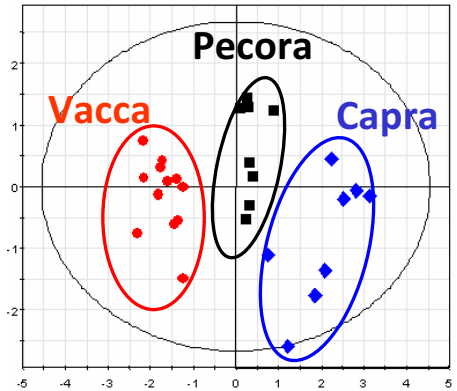
Determinazione della provenienza BOTANICA del miele



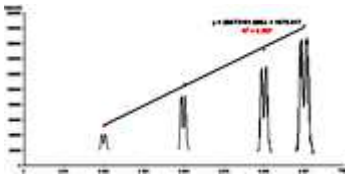
Determinazione della FILIERA DI PRODUZIONE del formaggio Asiago



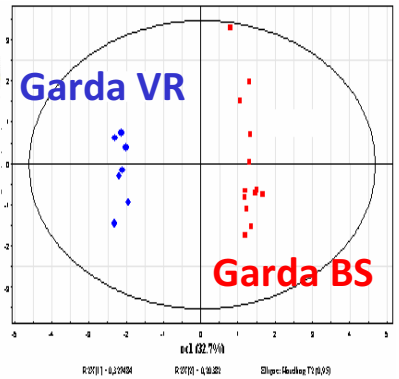
Determinazione della provenienza GENETICA delle caciotte



Determinazione QUANTITATIVA di metaboliti



Determinazione della provenienza GEOGRAFICA dell'olio d'oliva



Claudio Modesti
Gruppo Editoriale Geronimo, 2005

Perchè mai usare l'NMR???

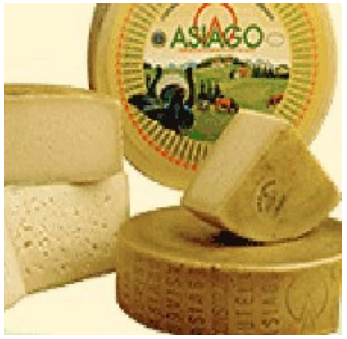
La competizione:

I vantaggi:

- pochi trattamenti del campione
- analisi in miscela
- velocità (spesso)
- <u>caratterizzazione di sostanze</u>

I tre problemi dell'NMR:

- sensibilità
- sensibilità
- sensibilità


ASIAGO

TRENTO
BELLUNO
ASIAGO
TREVISO
VICENZA
VERONA
PADOVA
VENEZIA
ROVIGO

PRODOTTO
DELLA
MONTAGNA

Asiago d'allevo "vecchio" (12 mesi)

Campionatura durante un intero anno:

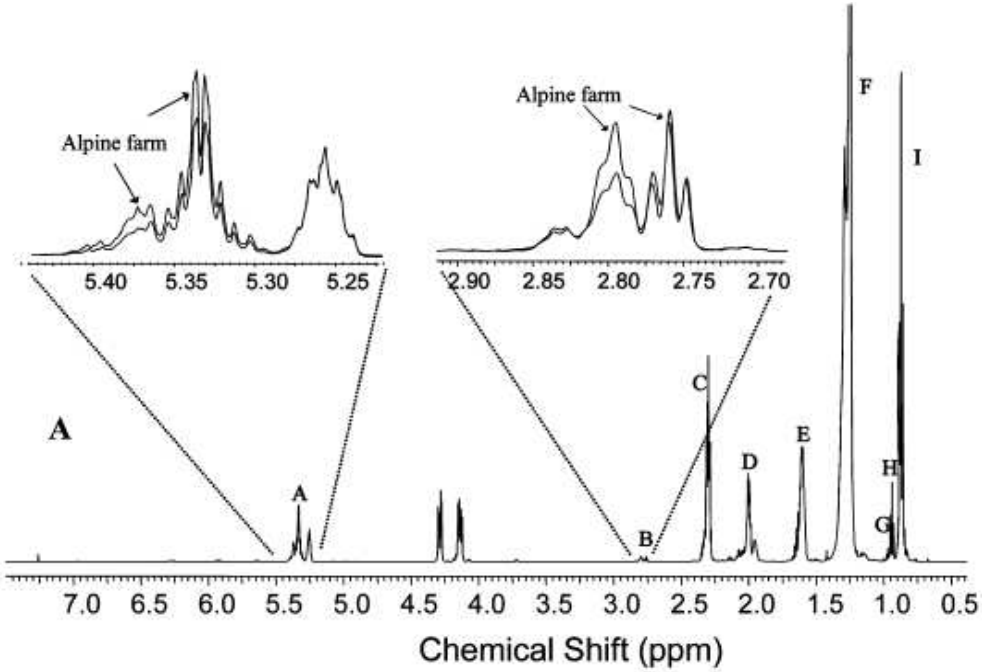
Malghe:	22 campioni
Caseifici di pianura:	15 campioni
Caseifici di montagna:	10 campioni

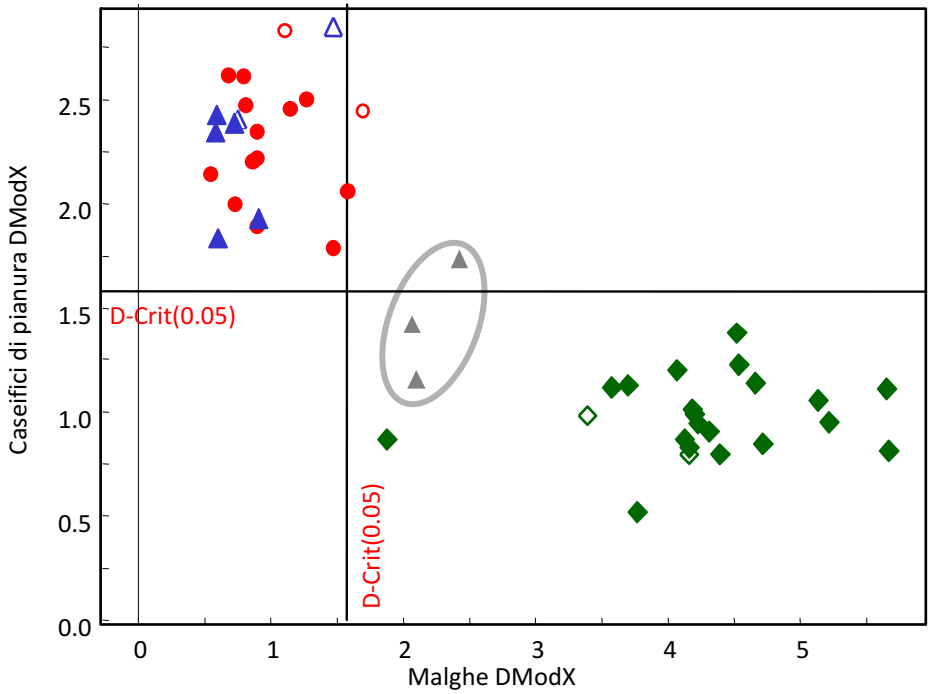
Un metodo di estrazione estremamente semplice:

- 0.2 g di formaggio grattugiato in una siringa da 5 mL
- aggiunta di 1 mL di $CDCl_3$
- agitazione della sospensione per 10 minuti
- filtrazione su membrana da 0.2 µm direttamente nel tubo NMR

E. Schievano, G. Pasini, G. Cozzi, S. Mammi
J. Agric. Food Chem. **2008,** *56,* 7208–7214

^{1}H – COMPONENTI MAGGIORITARIE

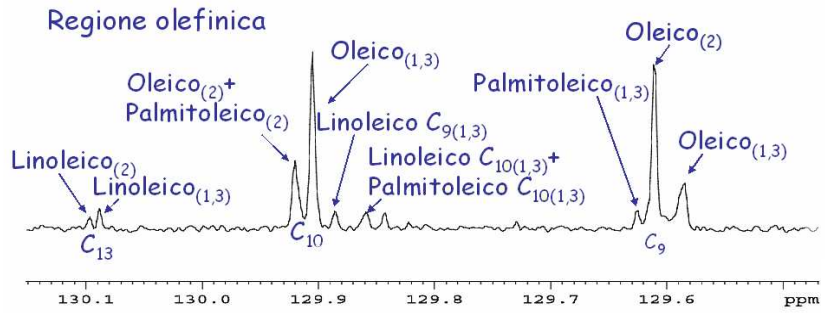


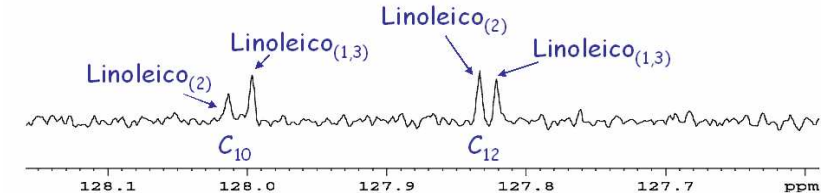


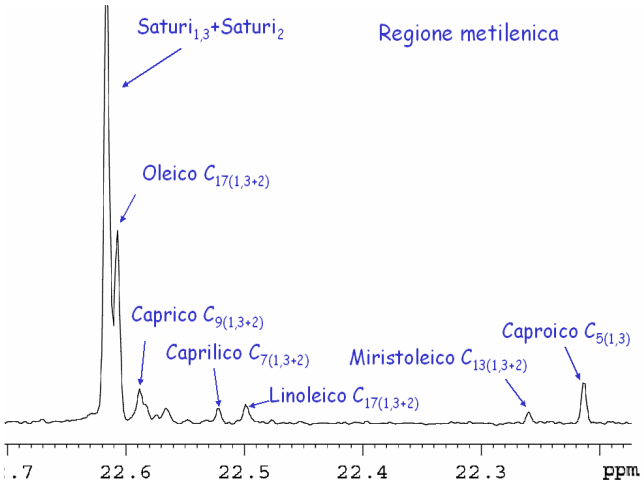
◆Malghe
●Caseifici di pianura
▲Caseifici di montagna

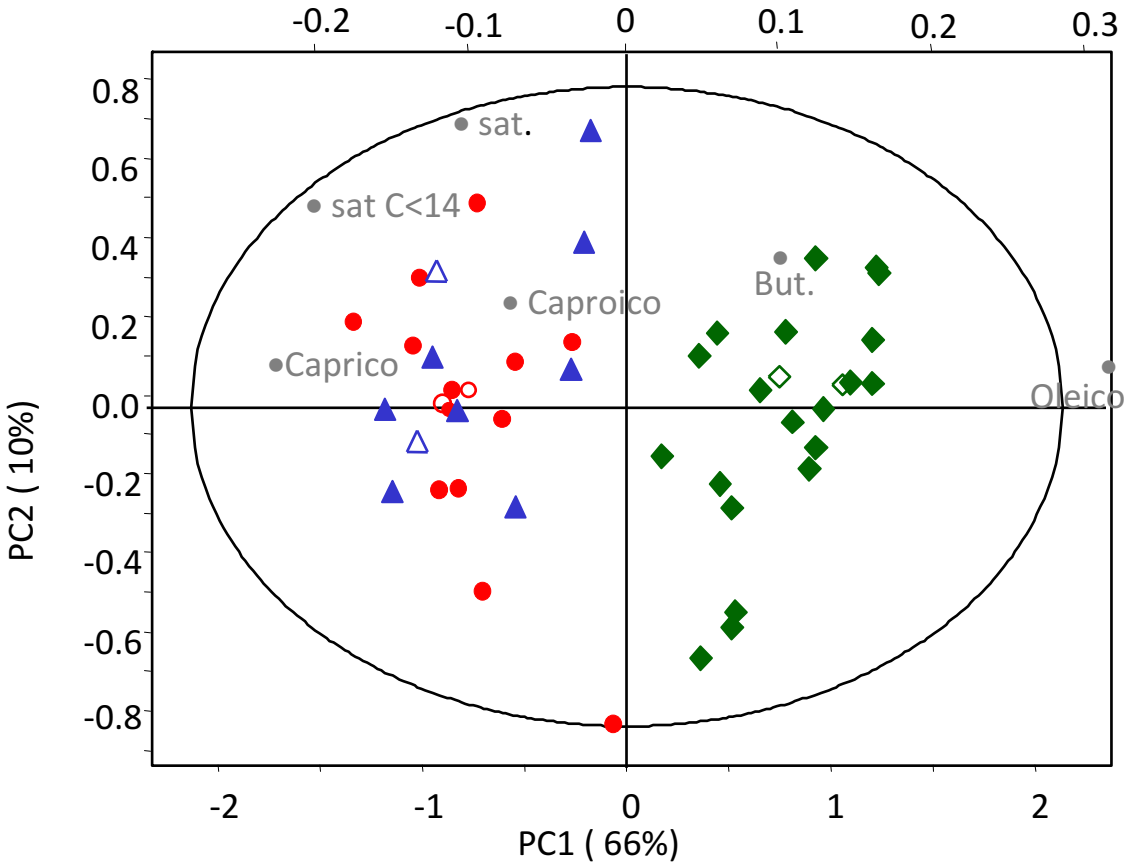
E. Schievano, G. Pasini, G. Cozzi, S. Mammi
J. Agric. Food Chem. **2008,** *56,* 7208–7214

^{13}C – COMPONENTI MAGGIORITARIE



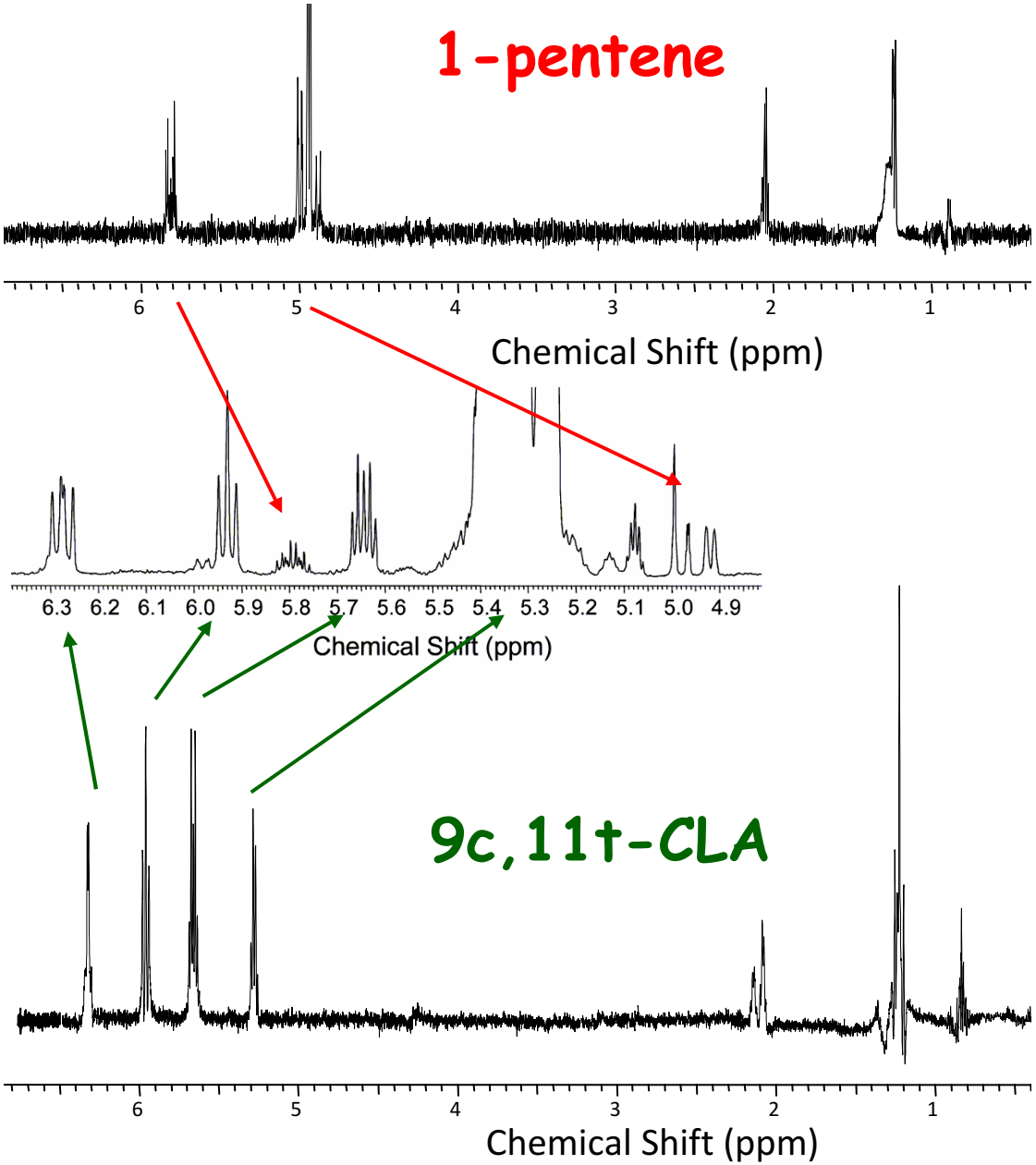




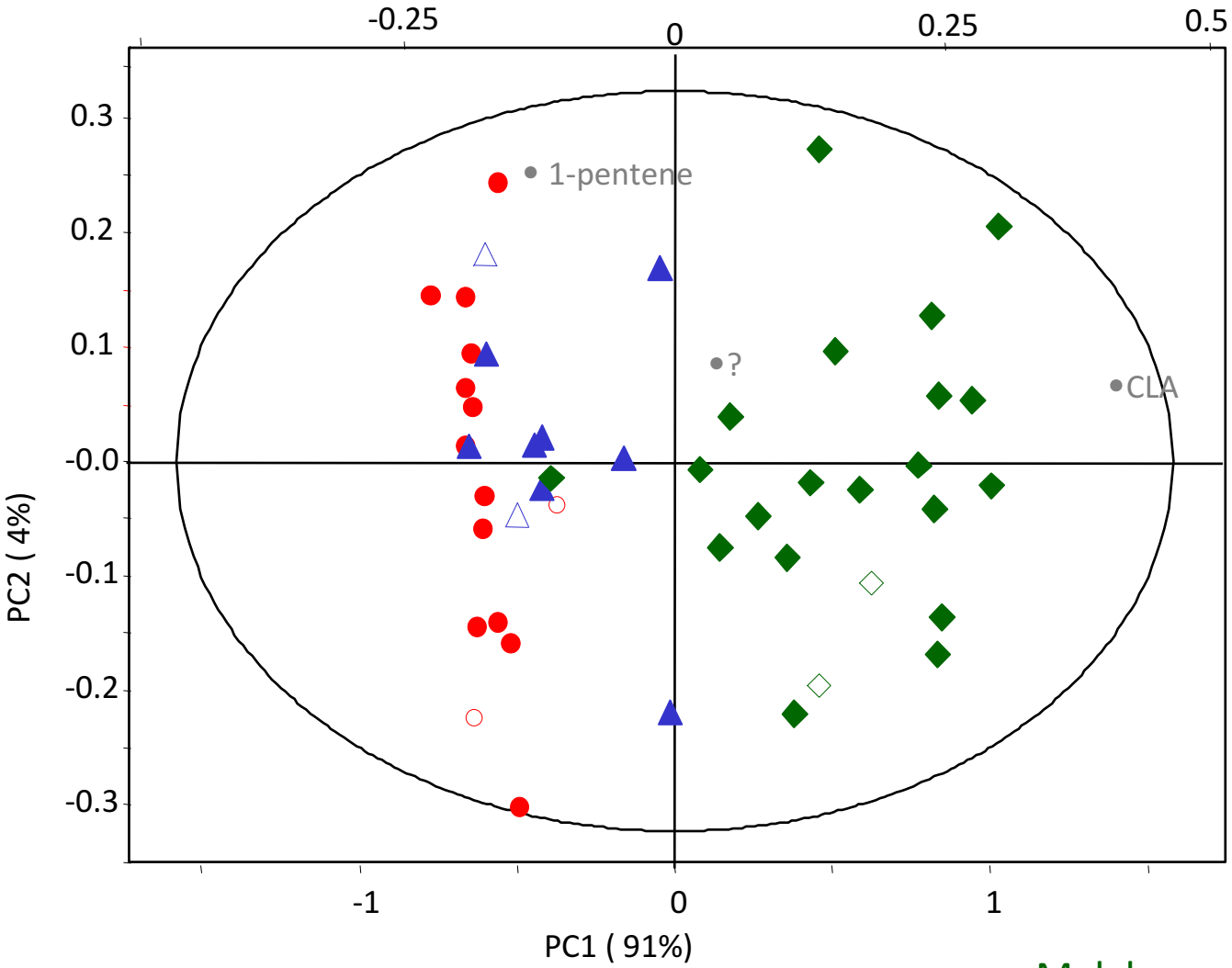


E. Schievano, G. Pasini, G. Cozzi, S. Mammi
J. Agric. Food Chem. **2008,** *56,* 7208–7214

^{1}H – COMPONENTI MINORITARIE



^{1}H – COMPONENTI MINORITARIE



◆ Malghe
● Caseifici di pianura
▲ Caseifici di montagna

E. Schievano, G. Pasini, G. Cozzi, S. Mammi
J. Agric. Food Chem. **2008,** *56,* 7208–7214

FEASR

REGIONE DEL VENETO

Fondo europeo agricolo per lo sviluppo rurale: l'Europa investe nelle zone rurali

Progetto UNIMIELE

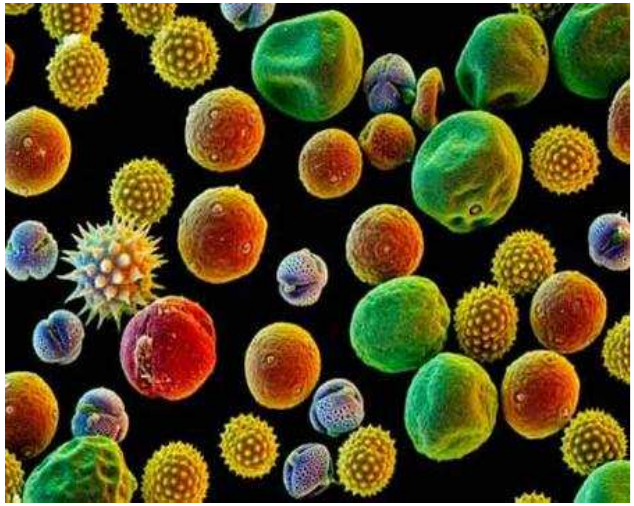
Reg. (CE) n. 1698/2005 - PSR 2007 – 2013 DGR n. 199 del 12/02/08

E. Schievano, E. Peggion, S. Mammi
J. Agric. Food Chem. 2010, 58, 57–65

E. Schievano, M. Stocchero, E. Morelato, C. Facchin, S. Mammi
Metabolomics 2012, 8, 679–690.

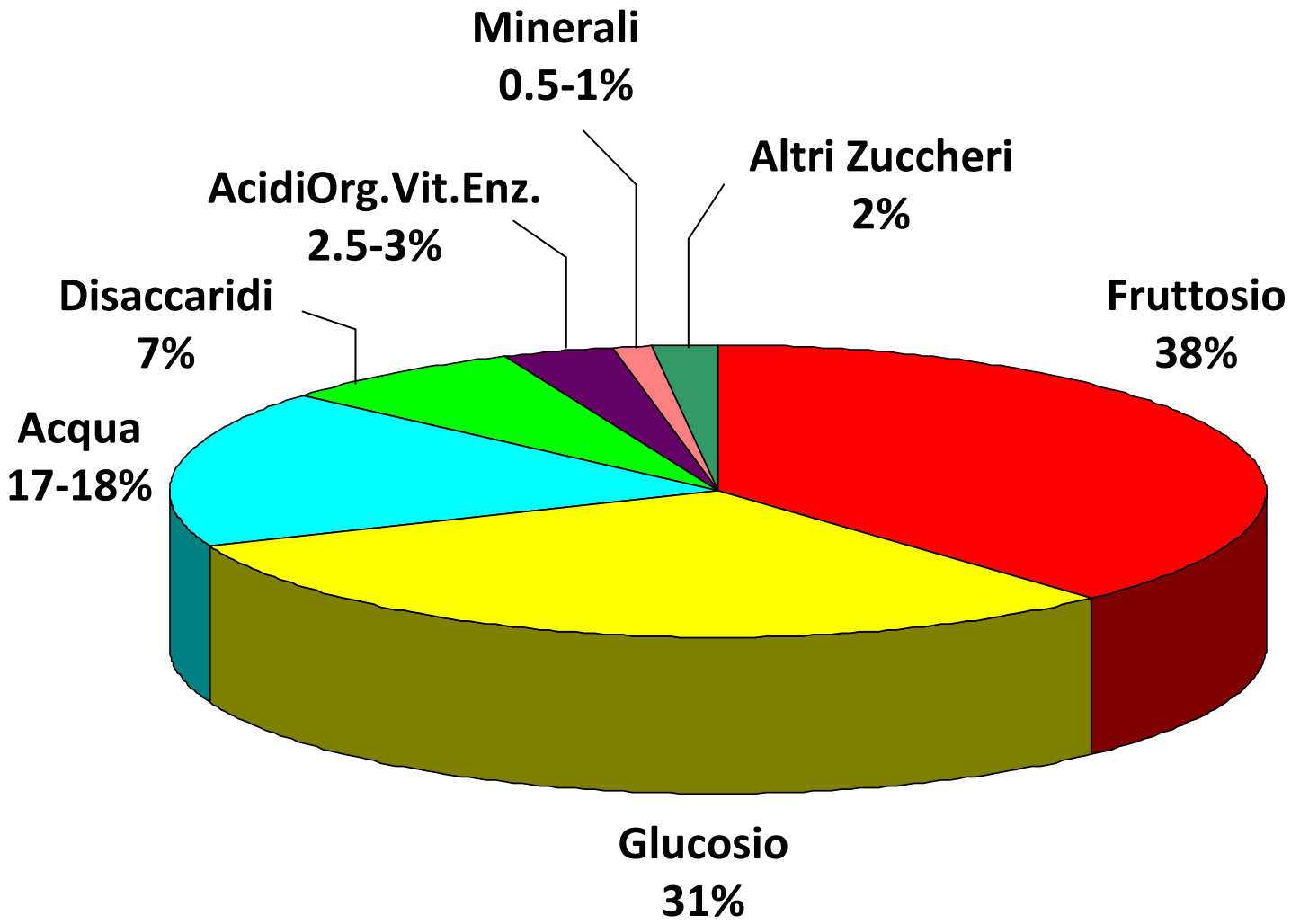
Analisi melissopalinologica

- Tecnica in uso per l'analisi del miele.
- Osservazione mediante microscopio dei diversi granuli pollinici contenuti nel miele.
- Tecnica non semplice e difficilmente standardizzabile e riproducibile: variazioni degli spettri pollinici dovute a diverse cause.



I limiti di questa tecnica applicata all'identificazione dell'origine botanica hanno portato alla ricerca di nuove tecniche in grado di dare risposte più esaurienti e precise.

Composizione Media del Miele



Procedura di estrazione

1 - Se il miele è cristallizzato, viene reso fluido attraverso un breve processo di riscaldamento a microonde (qualche secondo), ponendo particolare attenzione a non superare i 35 °C.

2 - 6 g di miele in una provetta di teflon da centrifuga e si aggiungono 15 mL di H_2O distillata. Si agita vigorosamente con agitatore meccanico per 15 minuti.

3 - Si aggiungono 15 mL di $CHCl_3$, si agita con agitatore meccanico per 15 minuti.

4 - L'emulsione che si forma dopo l'agitazione viene rotta mediante centrifuga per 15 minuti a 20 °C con velocità di 10000 rpm.

5 - Si raccoglie la parte organica sottostante in provetta di vetro e la si pone ad essiccare sotto flusso di azoto.

6 - Si scioglie in 600 mL di $CDCl_3$ e si procede con l'analisi ponendo questa quantità in un tubo NMR da 5 mm.

E. Schievano, E. Peggion, S. Mammi
J. Agric. Food Chem. 2010, 58, 57–65

Origine botanica	
ACACIA	79
CASTAGNO	76
TIGLIO	81
MILLEFIORI	48
ARANCIO	55
EUCALIPTO	56
MELATA	56
LIMONE	4
TRIFOGLIO	1
MELO	4
MONTAGNA	9
SULLA	3
TIMO	1
GIRASOLE	1
TOTALE	**474 (451)**

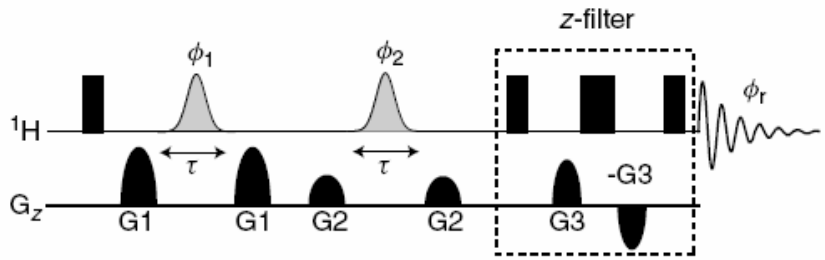
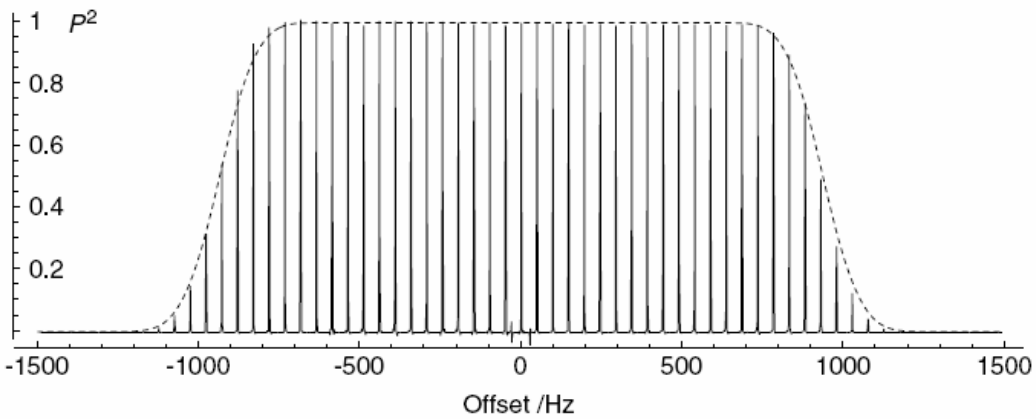
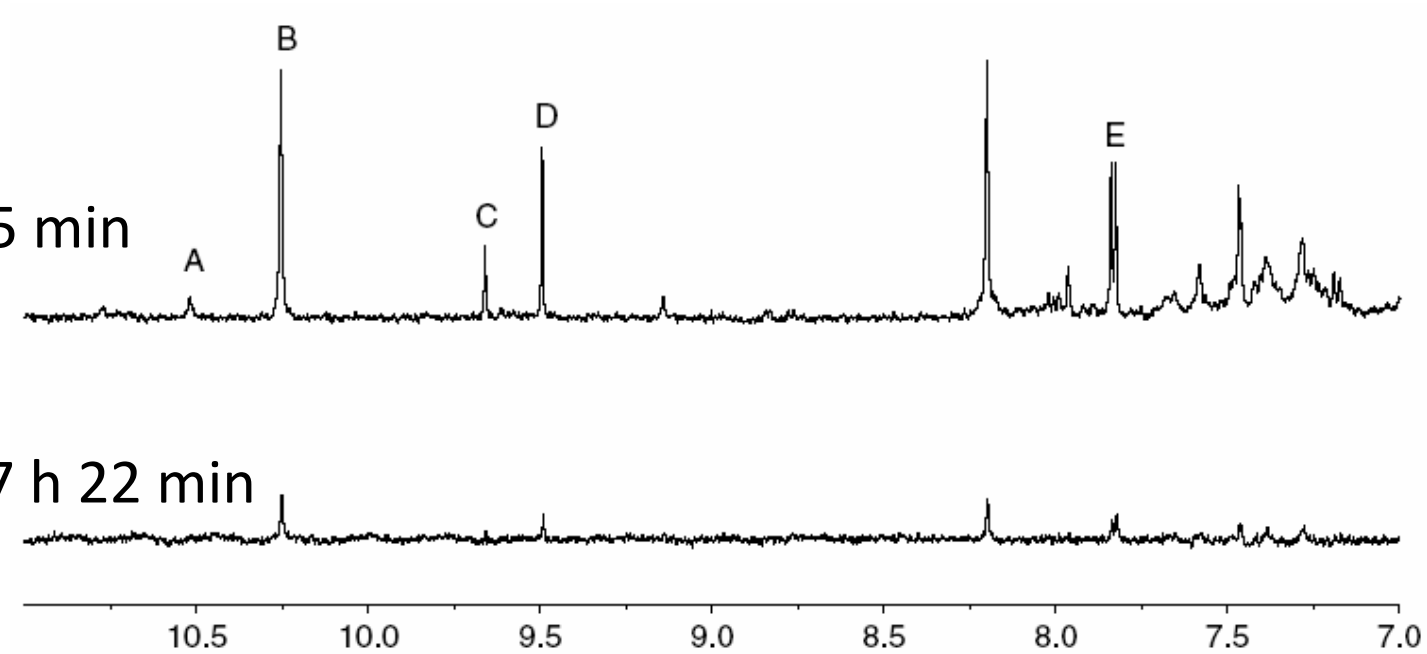


Figure 1. DPFGSE pulse scheme with optional *z*-filter to remove anti-phase magnetisation (see Supporting Information). Adiabatic pulses of duration τ are represented by their amplitude envelope (in grey). Black rectangles represent pulses of 90° (thin) and 180° (thick) flip angles with phase *x*; Exorcycle on ϕ_1 and ϕ_r is nested within a CYCLOPS phase cycling.



512 scans, 45 min

4096 scans, 7 h 22 min



F. Rastrelli, E. Schievano, A. Bagno, S. Mammi
Magn. Reson. Chem. 2009, *47*, 868–872

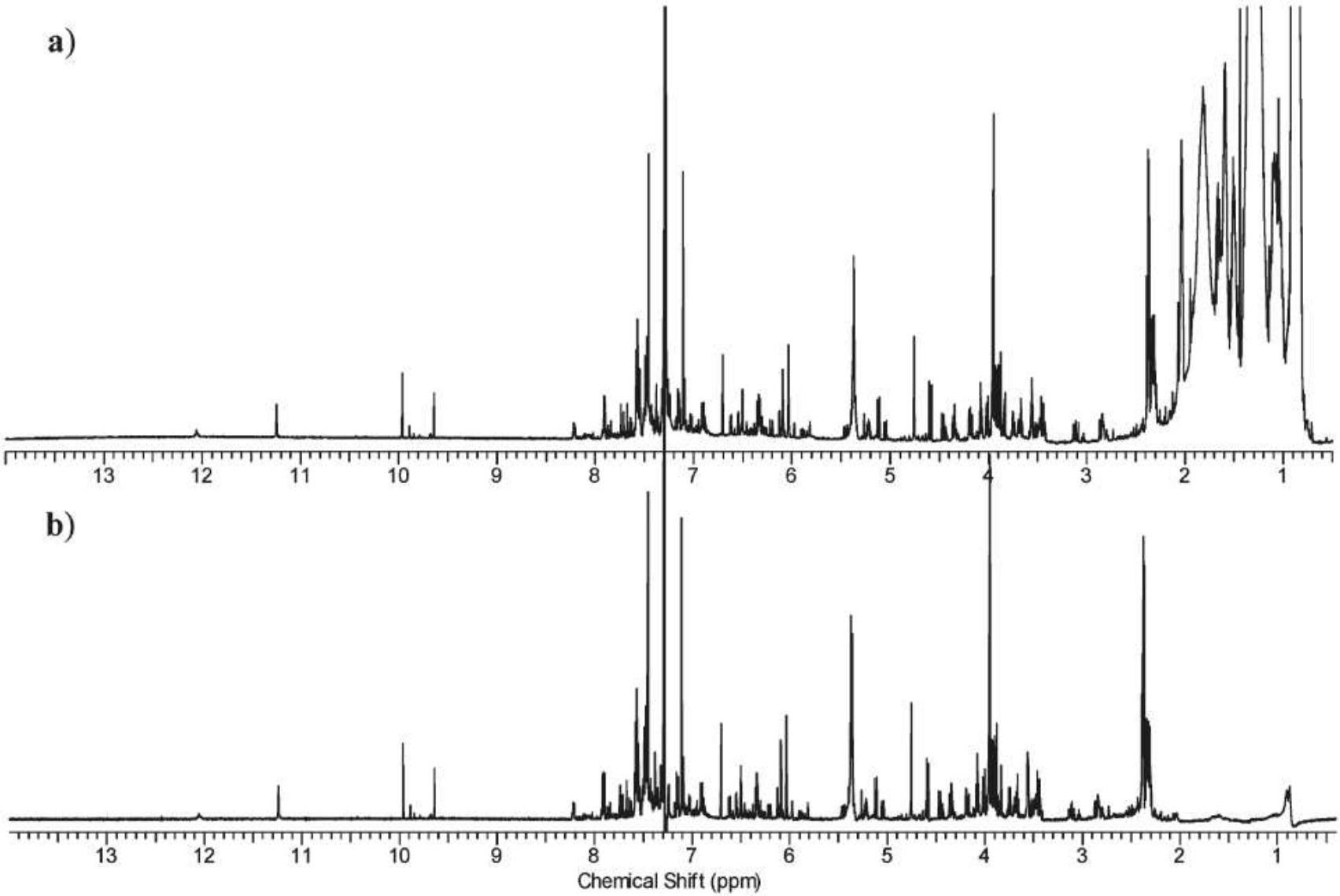
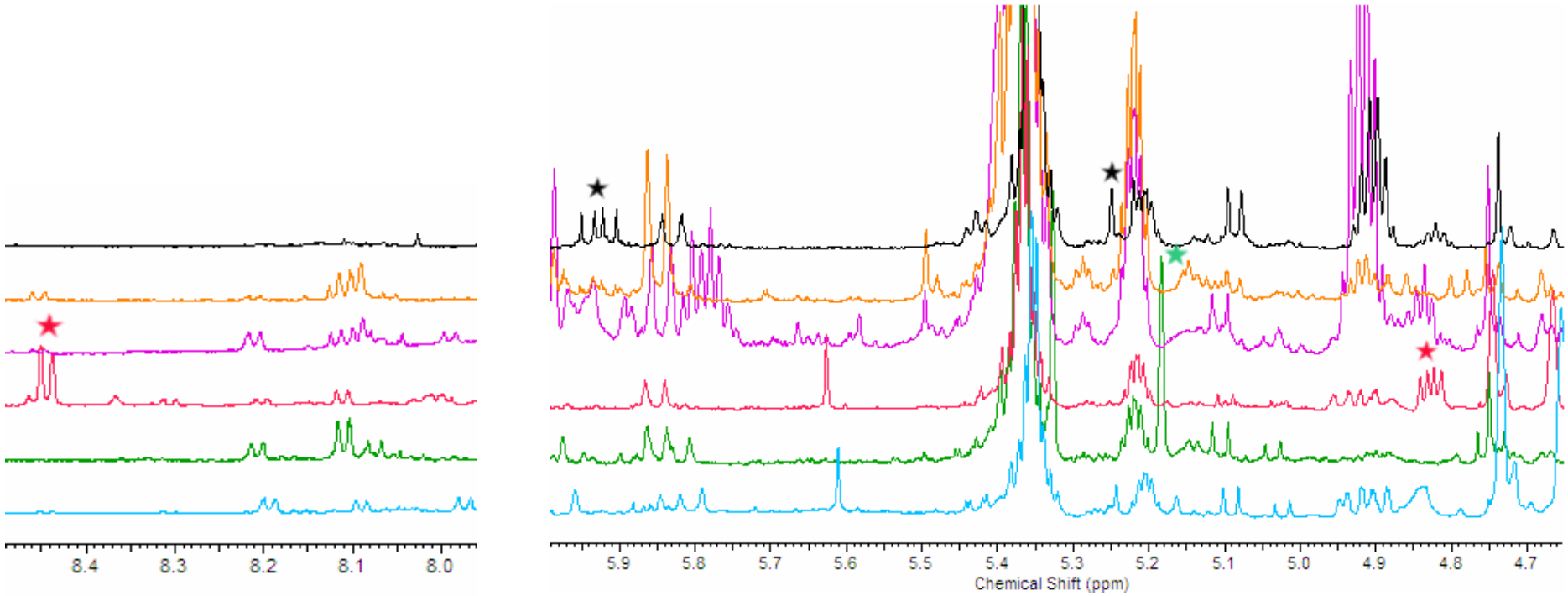


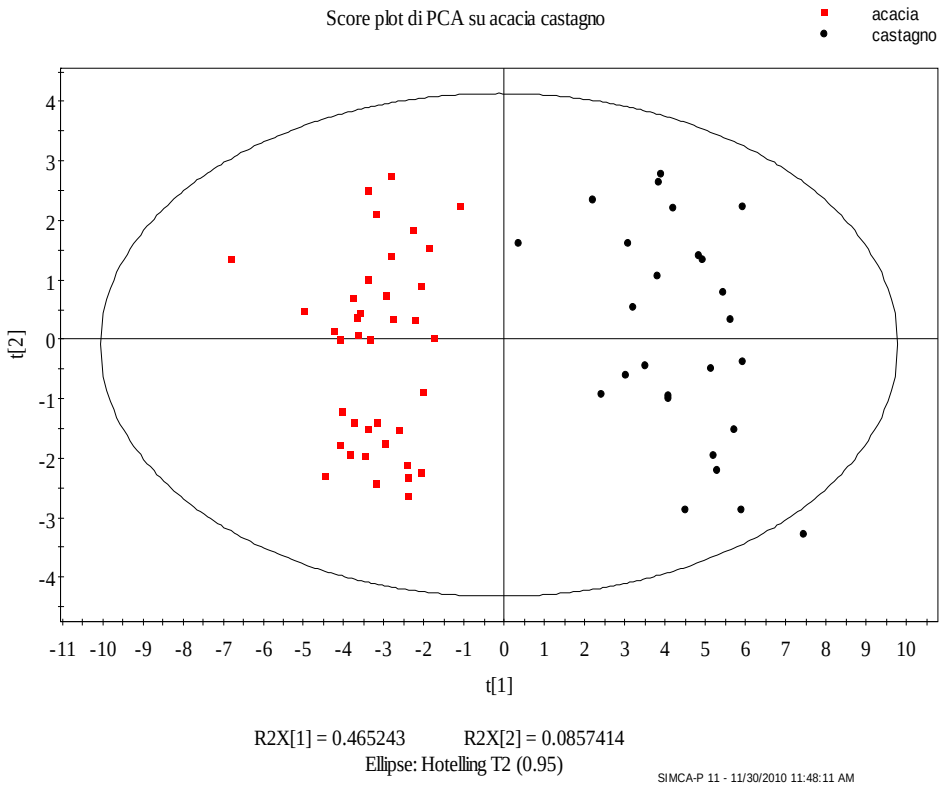
Figure 1. Comparison between complete 1D ^{1}H spectra of a honey sample obtained with (**a**) a single pulse sequence in 50 min and (**b**) the modified DPFGSE sequence in 25 min.

E. Schievano, E. Peggion, S. Mammi
J. Agric. Food Chem. 2010, 58, 57–65

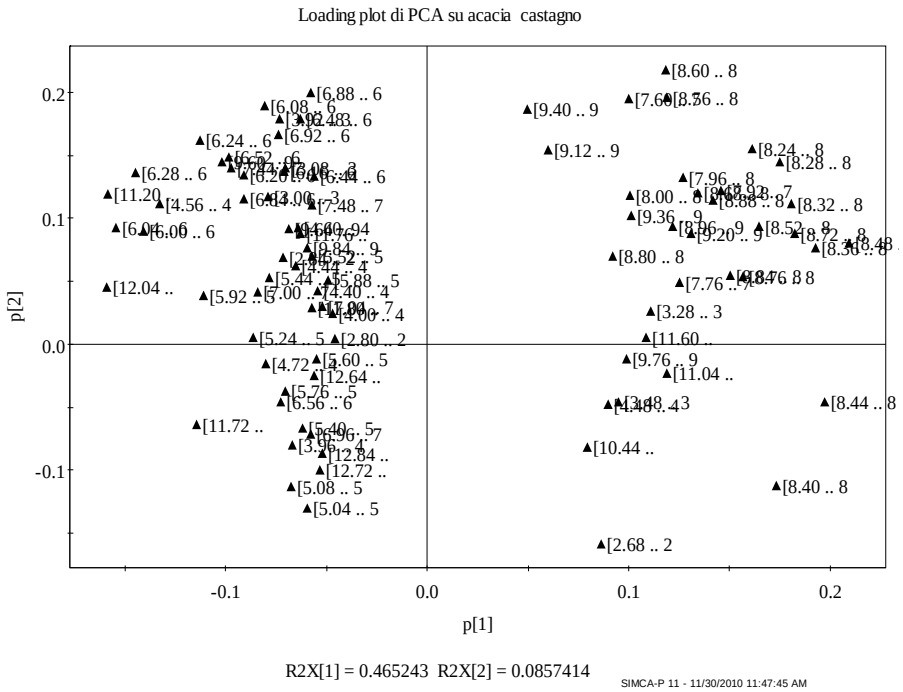
Spettri di mieli unifloreali



ANALISI DELLE COMPONENTI PRINCIPALI



Campioni



Variabili

Nella fase 1 del modello è stata eseguita una PCA separatamente su campioni appartenenti a ciascuna tipologia di miele. Questa analisi serve per evidenziare eventuali outliers *e per selezionare da ciascuna PCA, mediante la tecnica di campionamento "*onion *D*-optimal design*", circa il 70% delle osservazioni (242 campioni) per costruire il* training set*. La restante parte dei campioni (111 campioni) è stata utilizzata come* test set*.*

Specie botanica	**N° campioni *training set***	**N° campioni *test set***
ARANCIO	27	12
CASTAGNO	42	18
ACACIA	52	21
TIGLIO	43	18
EUCALIPTO	20	12
MELATA	25	11
MILLEFIORI	33	15

Totale 353 (349)

L'analisi ha evidenziato la presenza di 4 campioni che possono definirsi *outliers*, 1 per la classe arancio, 1 per la classe tiglio e 2 per la classe acacia.

Fase 1

arancio | *acacia* | *castagno* | *eucalipto* | *tiglio* | *melata* | *millefiori*

- pattern recognition to highlight strong outliers definition of the applicability domain (T2 test and DModX test)

Fase 2

-given a new X_i, if $X_i \in domain(\text{classification model})$ *then* $\exists k : X_i \in domain(PCA_k)$

One versus all approach

Fase 3

arancio | *acacia* | *castagno* | *eucalipto* | *tiglio* | *melata* | *millefiori*

other classes | *other classes* | *other classes* | *other classes* | *other classes* | *other classes* | *other classes*

Naïve Bayes classifier

Fase 4

t_p | t_p | t_p | t_p | t_p | t_p | t_p

class

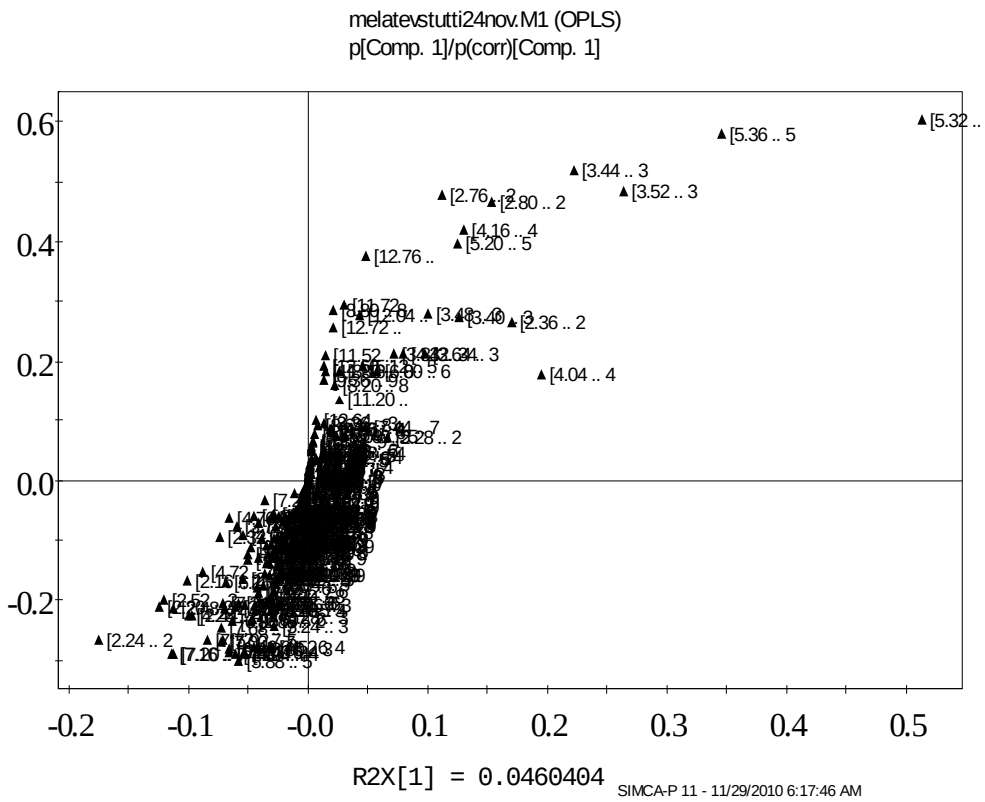
E. Schievano, M. Stocchero, E. Morelato, C. Facchin, S. Mammi
Metabolomics 2012, 8, 679–690.

Il training set è stato usato per costruire modelli *uno vs tutti* con un'analisi O2PLS-DA dove ogni classe di miele viene confrontato con le altre classi, a loro volta, messe tutte insieme in un'unica grande classe.

Con questo metodo si ottengono **7** modelli ciascuno con una componente predittiva che è stata usata per due scopi:

1-Predire la classe di campioni incogniti nel nostro caso del test set e altri 120 campioni acquisiti successivamente.

2-Ricerca dei potenziali marcatori sostanze presenti solo o in una quantità maggiore in un certo tipo di miele.



	P_Ar	P_Cast	P_AC	P_Ti	P_EU	P_Me	P_Mil	TOTAL	SENSITIVITY[%]
Arancio	**11**	0	0	0	0	0	1	**12**	**91.7**
Castagno	0	**16**	1	1	0	0	0	**18**	**88.9**
Acacia	0	0	**21**	0	0	0	0	**21**	**100.0**
Tiglio	0	1	0	**17**	0	0	0	**18**	**94.4**
Eucalipto	0	0	0	0	**12**	0	0	**12**	**100.0**
Melata	0	0	0	0	0	**10**	1	**11**	**90.9**
Millefiori	1	0	1	1	0	0	**12**	**15**	**80.0**
PRECISION[%]	**91.7**	**94.1**	**91.3**	**89.5**	**100.0**	**100.0**	**85.7**		

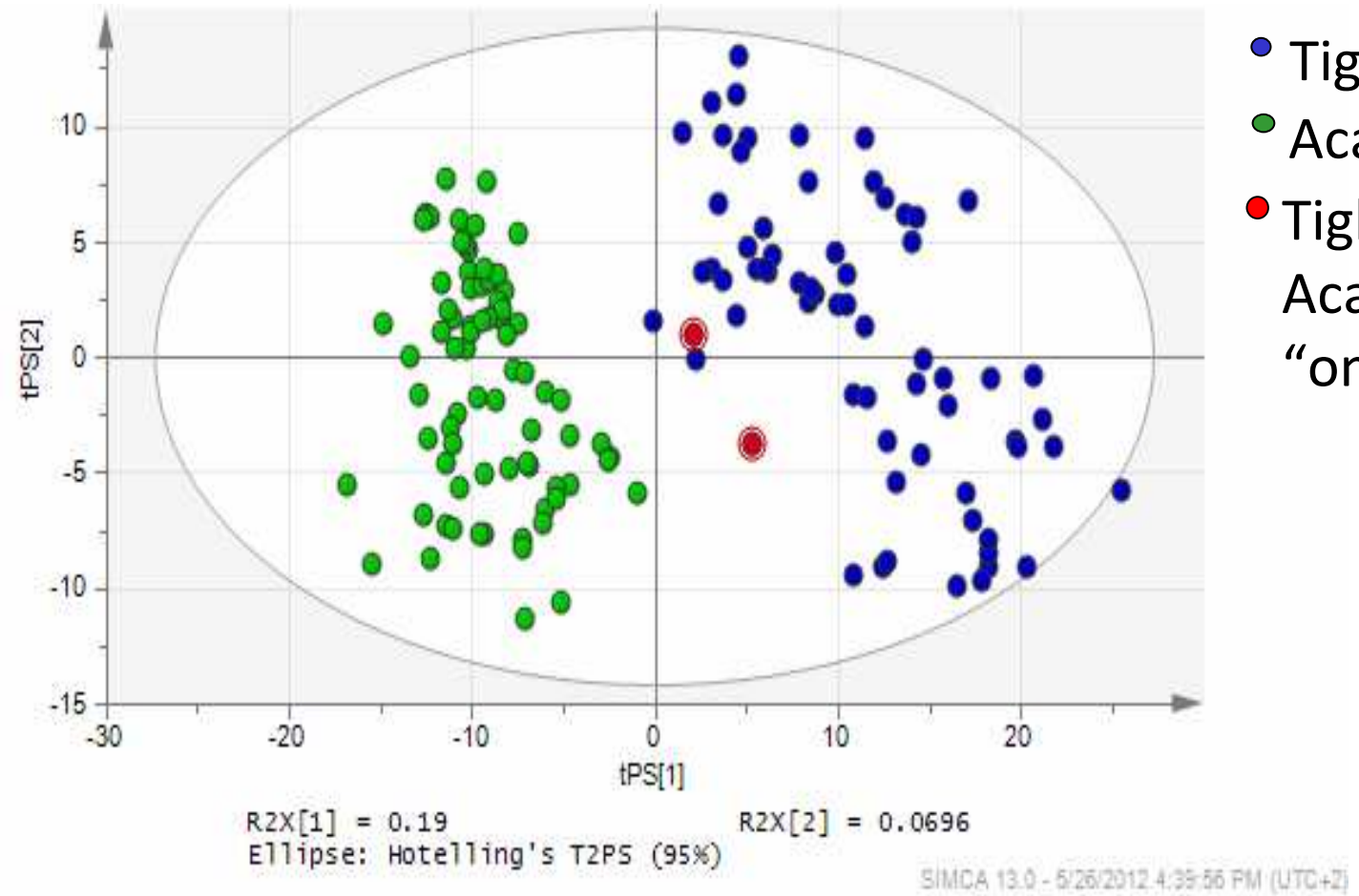
Sensitivity: ***per ogni specie botanica, è il rapporto percentuale tra i campioni correttamente predetti e il totale dei campioni appartenenti all'origine botanica considerata.***

Precision: ***è invece il rapporto percentuale tra i campioni correttamente predetti di quella classe e il totale di tutti i campioni del*** **dataset** ***assegnati a quella classe.***

Predizione su ulteriori 120 campioni con il modello “one vs all”

	P_Ar	P_Cast	P_AC	P_Ti	P_EU	P_Me	P_Mil	TOTAL	SENSITIVITY[%]
Arancio	**14**	0	1	0	0	0	2	**15**	**93.3**
Castagno	0	**15**	0	1	0	0	1	**17**	**88.2**
Acacia	0	0	**7**	0	0	0	5	**12**	**58.3**
Tiglio	0	1	2	**18**	0	0	2	**23**	**77.3**
Eucalipto	2	0	0	0	**22**	0	0	**24**	**91.6**
Melata	0	0	0	0	1	**20**	3	**24**	**83.3**
Millefiori	0	0	1	1	0	0	**3**	**5**	**60.0**
PRECISION[%]	**87.5**	**93.7**	**63.6**	**85.0**	**95.6**	**100.0**	**21.4**		

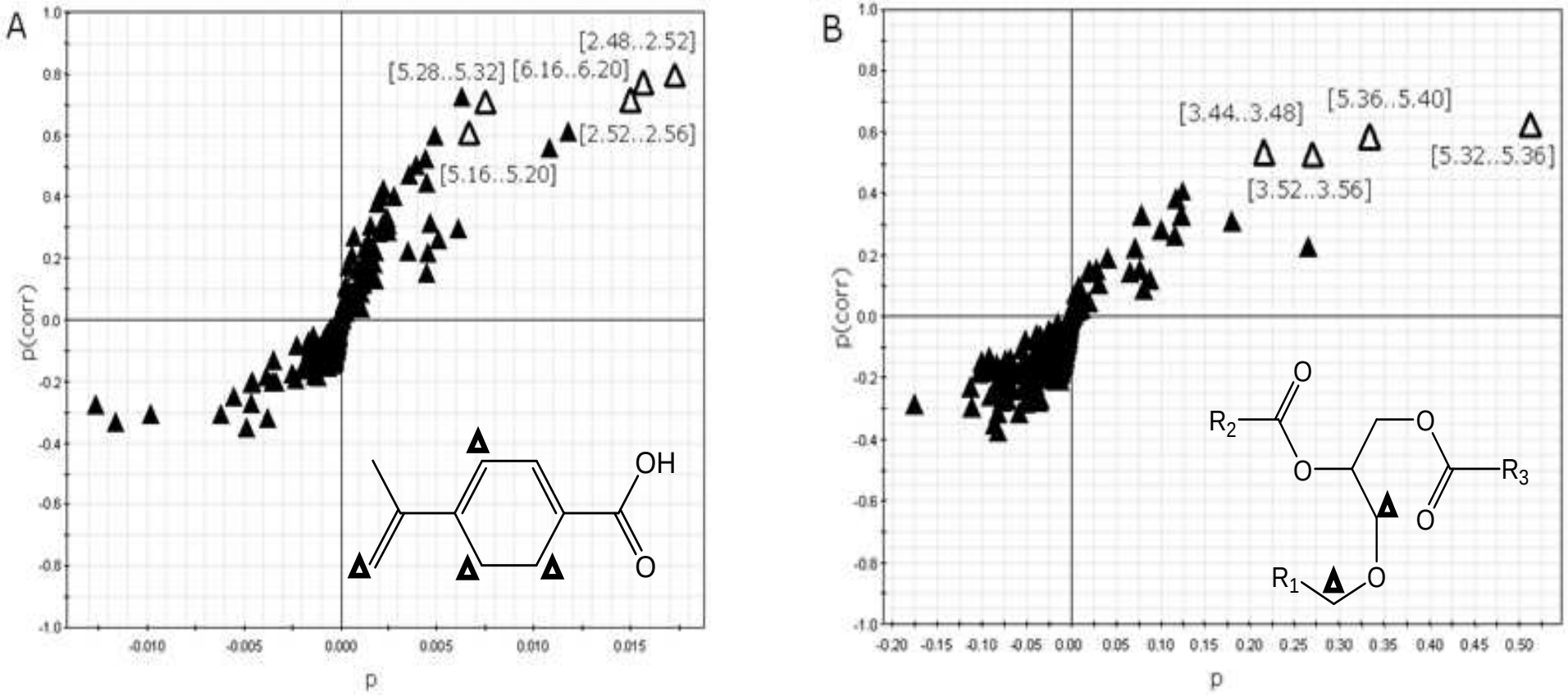
PLS-DA Acacia vs Tiglio



- Tiglio
- Acacia
- Tigli predetti Acacia dal modello "one vs all"

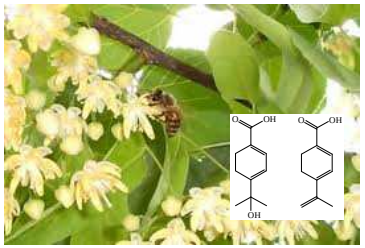
Predizione su ulteriori 120 campioni con il modello PLS-DA “one vs one” per gli incerti

	P_Ar	P_Cast	P_AC	P_Ti	P_EU	P_Me	P_Mil	TOTAL	SENSITIVITY[%]
Arancio	**15**	0	0	0	0	0	0	**15**	**100.0**
Castagno	0	**16**	0	1	0	0	0	**17**	**94.1**
Acacia	0	0	**9**	0	0	0	3	**12**	**75.0**
Tiglio	0	1	0	**20**	0	0	2	**23**	**86.9**
Eucalipto	2	0	0	0	**22**	0	0	**24**	**91.6**
Melata	0	0	0	0	0	**22**	2	**24**	**91.6**
Millefiori	0	0	0	1	0	0	**4**	**5**	**80.0**
PRECISION[%]	**88.2**	**94.1**	**100.0**	**91.3**	**100.0**	**100.0**	**36.4**		



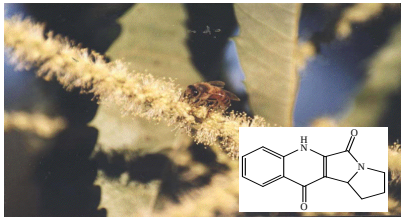
Esempi di S-plots per I modelli O2PLS-DA usati per caratterizzare le classi Tiglio (A) e Melata (B). I segnali dei markers putativi sono disegnati come triangoli aperti, sia nell'S-plot che sulla struttura della molecola.

E. Schievano, M. Stocchero, E. Morelato, C. Facchin, S. Mammi
Metabolomics 2012, 8, 679–690.

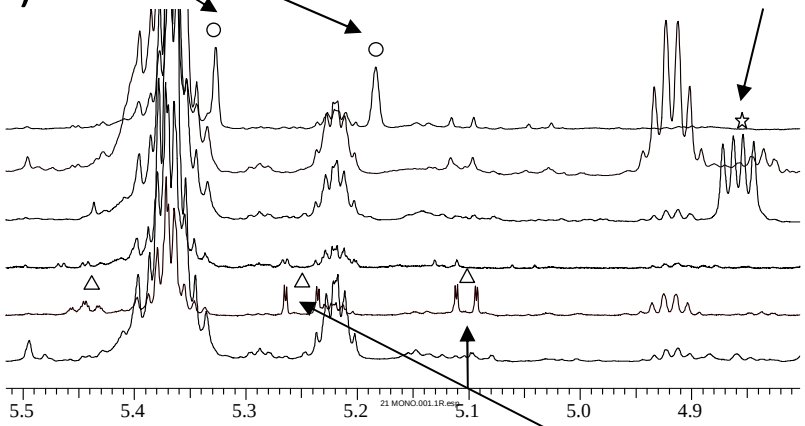


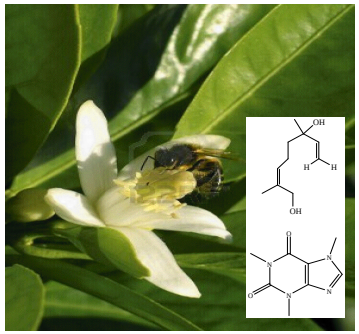
Tiglio

4-(1-hydroxy-1-methylethyl) cyclohexa-1,3-diene-carboxylic acid
and 4-(1-methylethenyl) cyclohexa-1,3-diene-carboxylic acid



Castagno

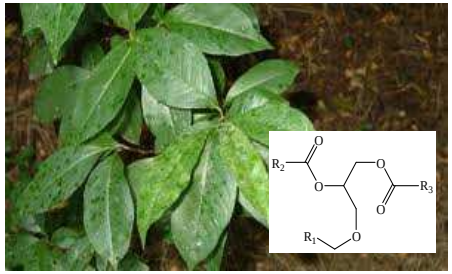




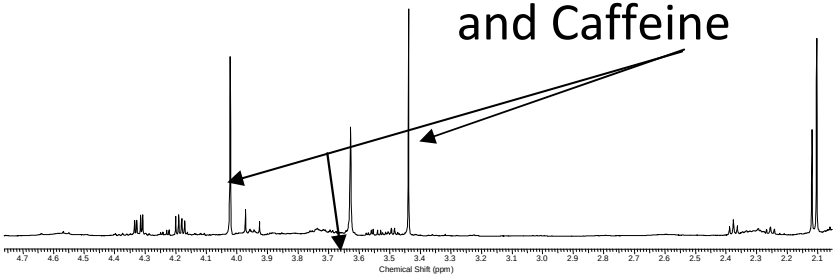
Arancio

8-hydroxylinalool and Caffeine

Diacylglycerylether

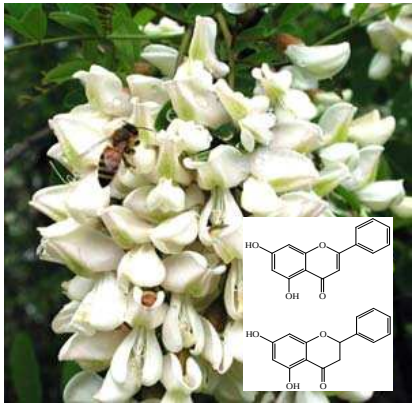


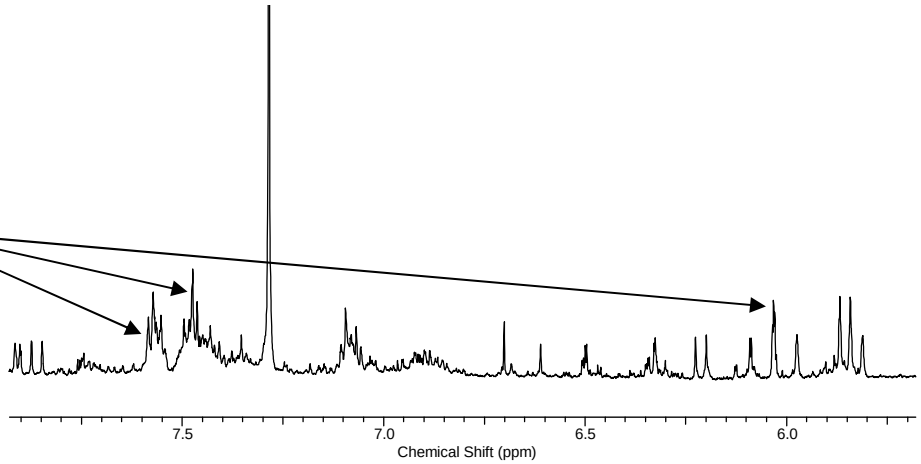
Melata



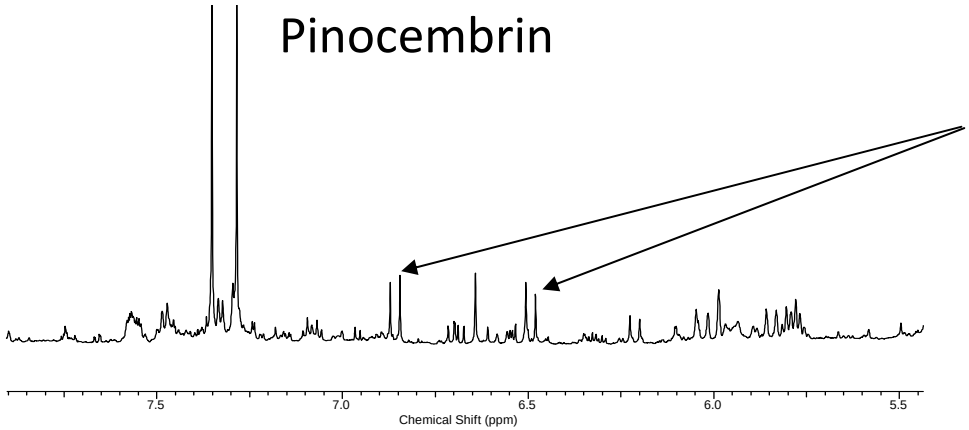
E. Schievano, M. Stocchero, E. Morelato, C. Facchin, S. Mammi
Metabolomics 2012, 8, 679–690.

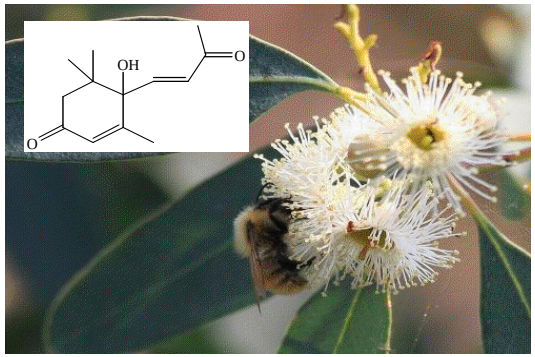
Acacia





Chrysin and
Pinocembrin





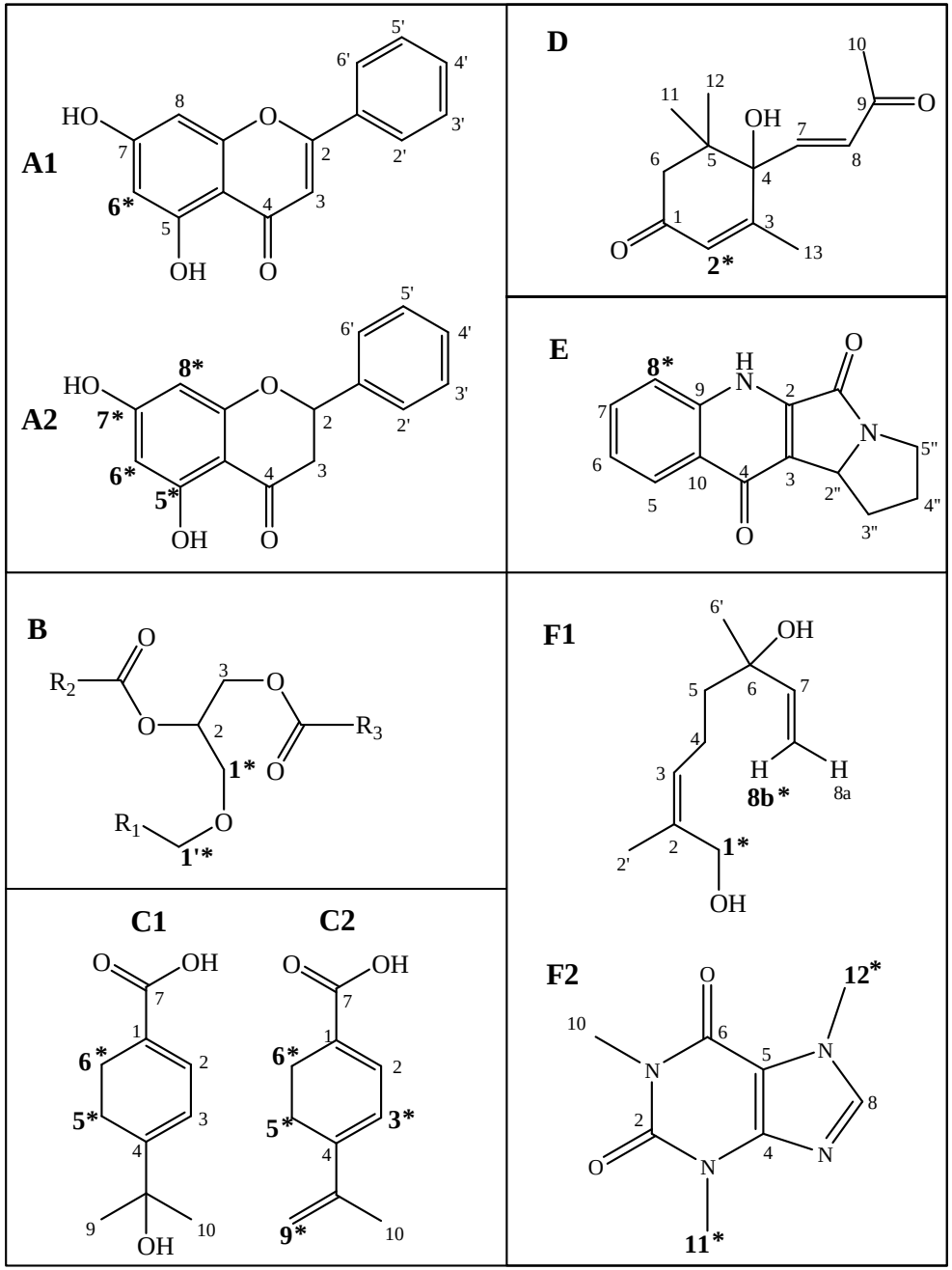
Eucalipto
Dehydrovomifoliol

E. Schievano, M. Stocchero, E. Morelato, C. Facchin, S. Mammi
Metabolomics 2012, 8, 679–690.

Acacia

Melata

Tiglio



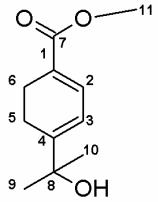
Eucalipto

Castagno

Arancio

E. Schievano, M. Stocchero, E. Morelato, C. Facchin, S. Mammi
Metabolomics 2012, 8, 679–690.

Tiglio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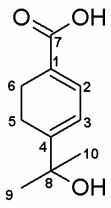
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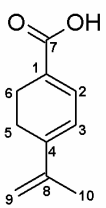
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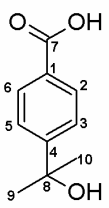
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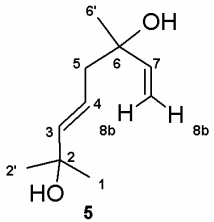
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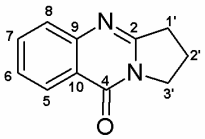
Tiglio/Castagno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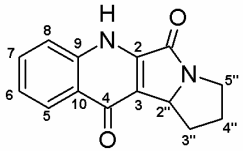
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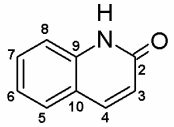
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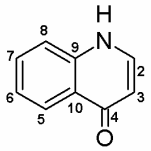
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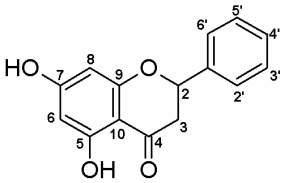
Castagno

Acacia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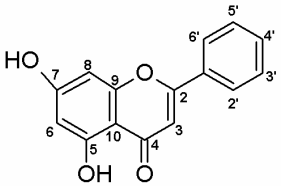
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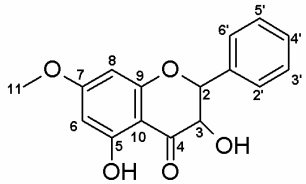
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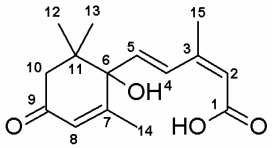
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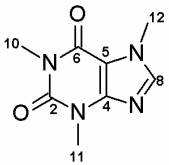
Arancio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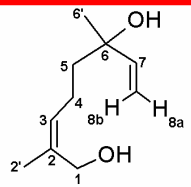
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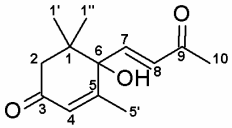
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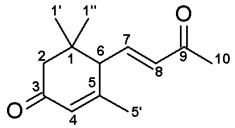
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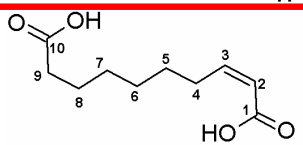
Eucalipto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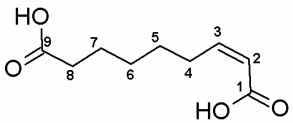
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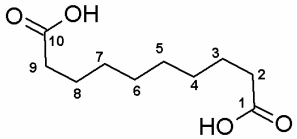
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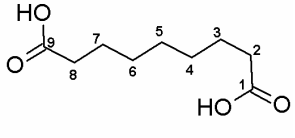
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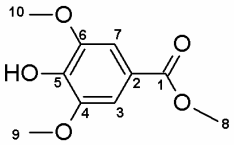
Prodotti dalle api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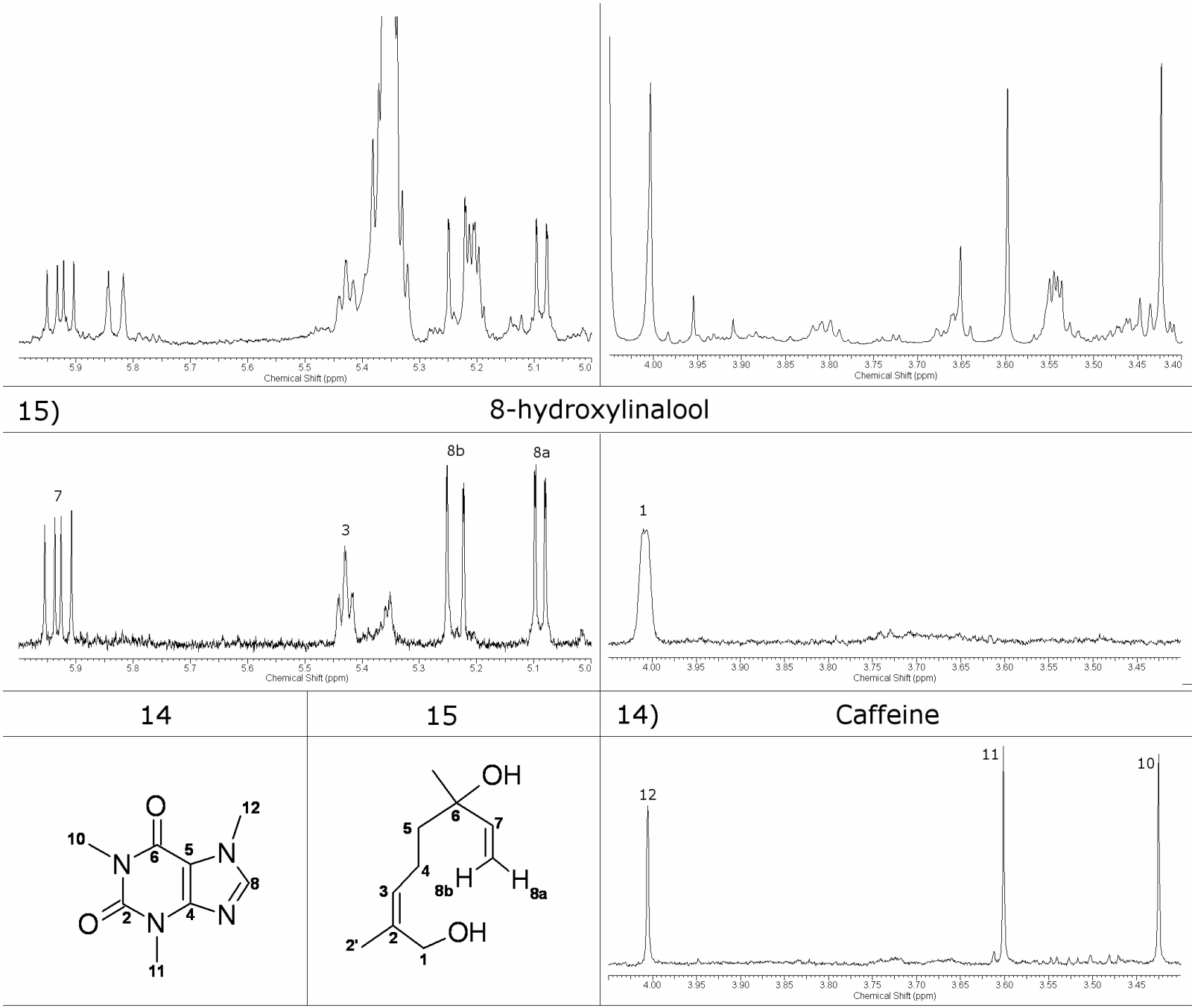
Presente in tutte le piante

E. Schievano, E. Morelato, C. Facchin, S. Mammi
J. Agr. Food Chem. 2013, 61, 1747-1755.



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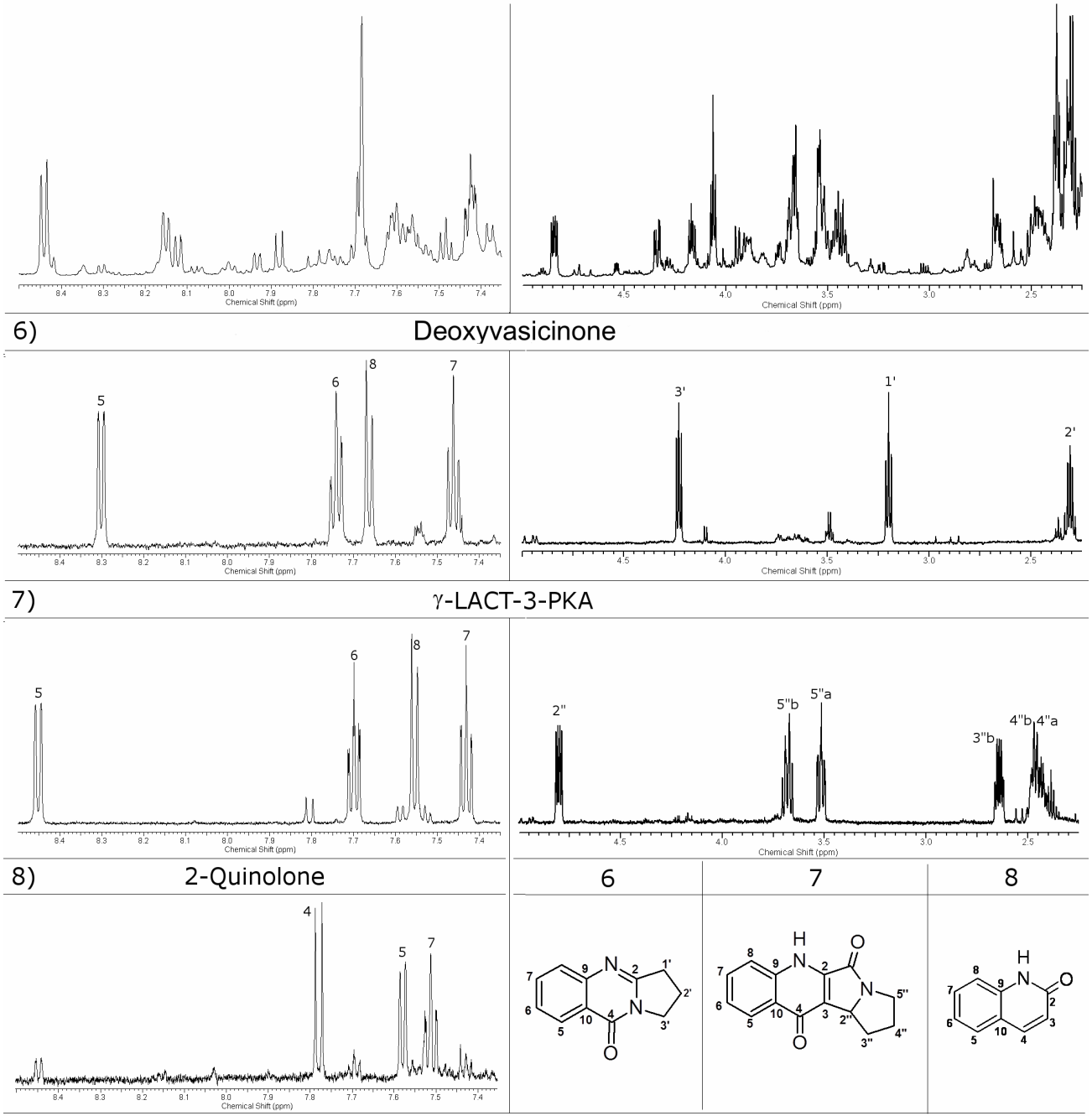

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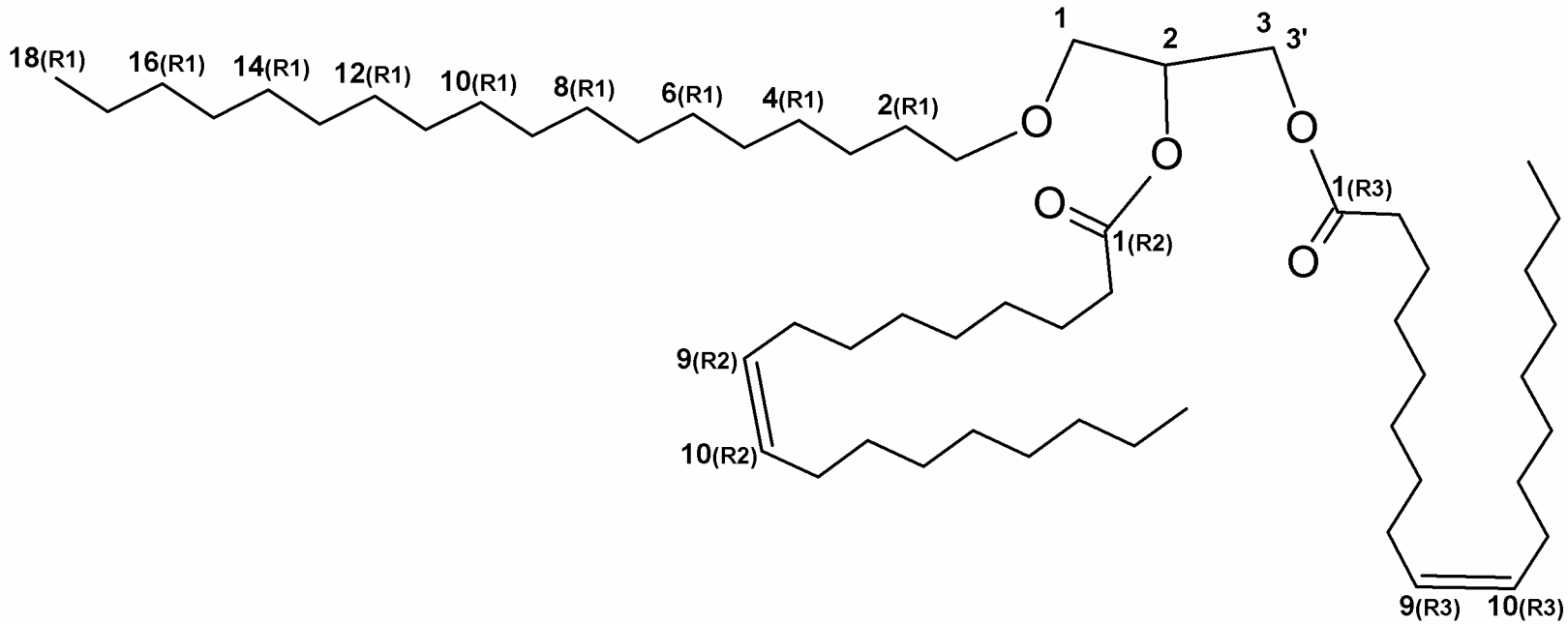


Melata

E. Schievano, E. Morelato, C. Facchin, S. Mammi
J. Agr. Food Chem. 2013, 61, 1747-1755.

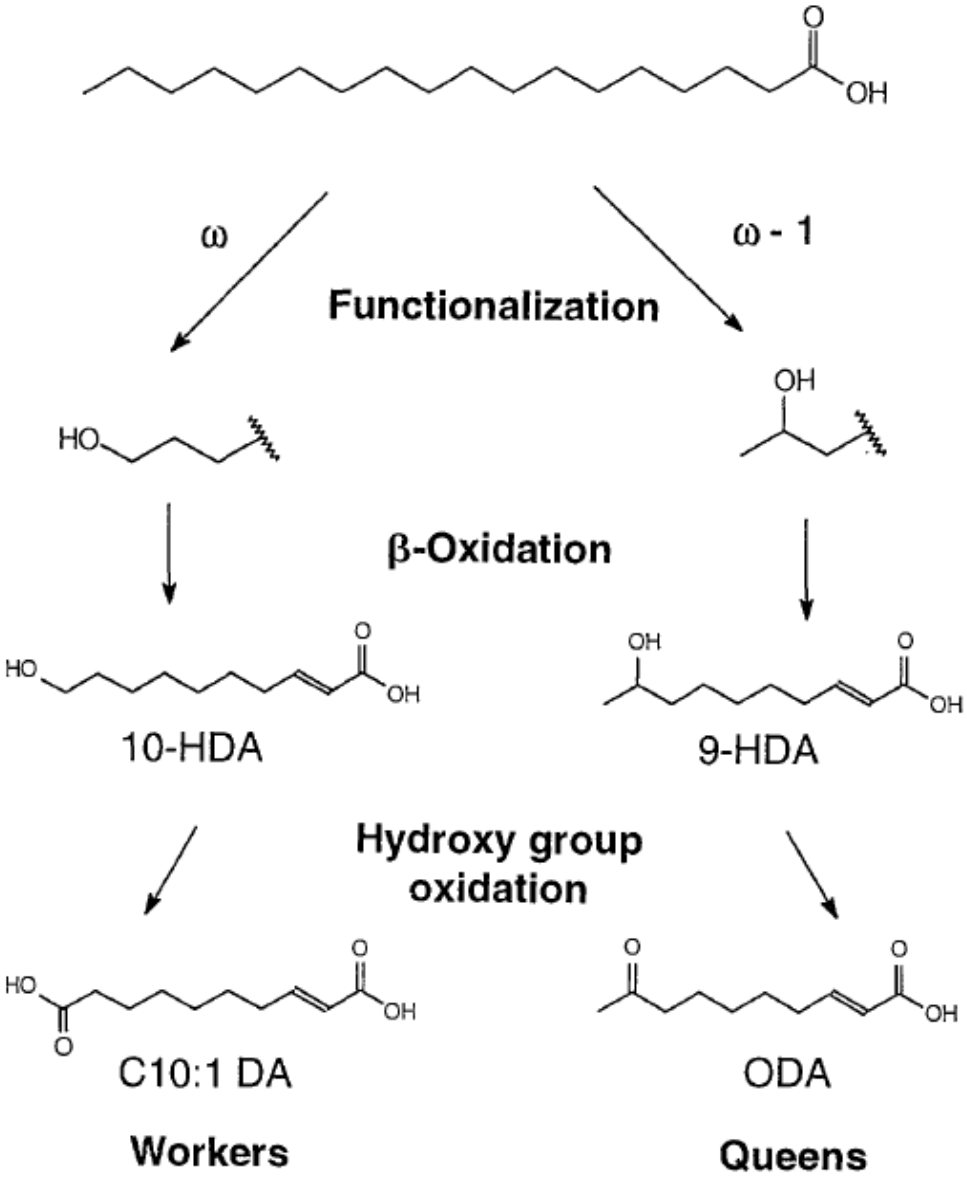


Fig. 1. Biosynthesis of ω- and (ω – 1)–functionalized 10-carbon acids from stearic acid in worker and queen honeybees. C10:1 DA, (*E*)2-decenedioic acid.

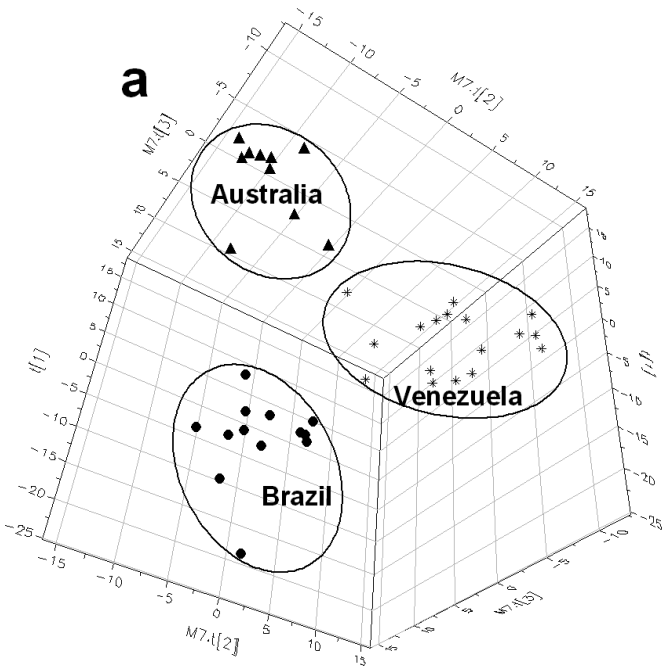
E. Plettner et al., SCIENCE 271, 1851 (1996)

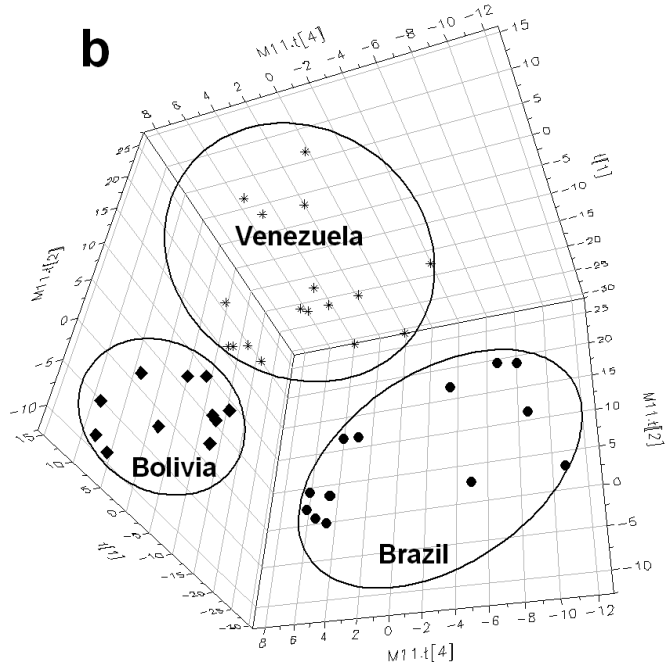
Apis mellifera

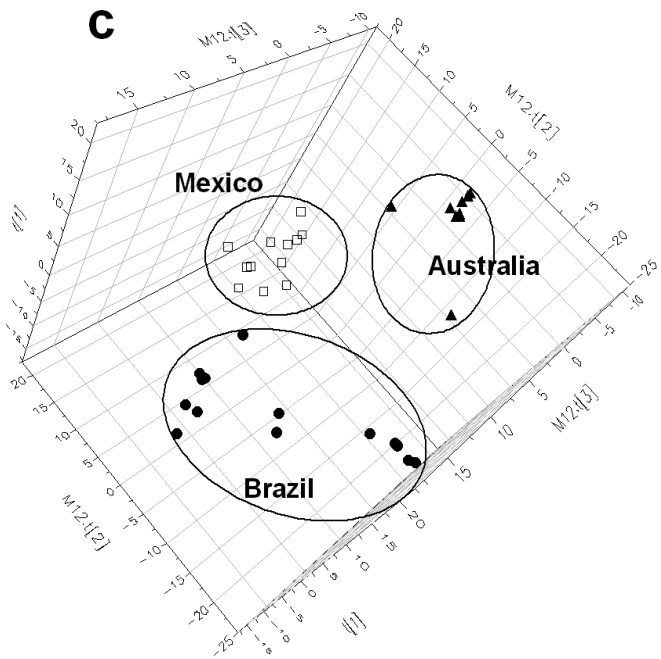
Melipona beecheii

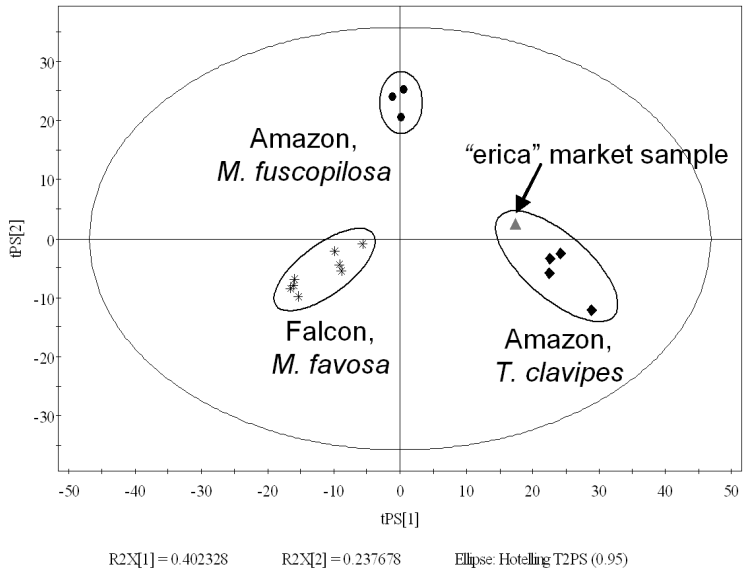
Melipona colimana

Common name	Stingless bee species	Geographical origin	Honey samples
"carby"	*Tetragonula carbonaria*	Australia, Brisbane	1-10
"uruçú"	*Melipona scutellaris*	Brazil, João Pessoa	11
"mijui"	*Scaptotrigona polysticta*	Brazil, Xingú	12
	Melipona sp.	Brazil	13
"tiúba"	*Melipona fasciculata*	Brazil, Maranhão	14-18
"jandaíra"	*Melipona subnitida*	Brazil, Rio Grande du Norte	19-22
"erica"	*Melipona favosa*	Venezuela, Falcón	23-30
"isabitto"	*Melipona fuscopilosa*	Venezuela, Amazon	31-33
"ajavitte"	*Tetragona clavipes*	Venezuela, Amazon	34-37
"pisilnekmej"	*Scaptotrigona mexicana*	Mexico	38-40
"colmena real"	*Melipona fasciata guerreroensis*	Mexico	41
"abeja real roja"	*Melipona fasciata guerreroensis*	Mexico	42
"criolla"	*Melipona solani*	Mexico	43
"abeja bermeja"	*Scaptotrigona hellwegeri*	Mexico	44-46
"ala blanca"	*Trigona nigra*	Mexico	47
"abeja real"	*Melipona beecheii*	Mexico	48-50
"erereú barcina"	*Melipona grandis*	Bolivia, Amboró National Park	51
"erereú choca"	*Melipona brachychaeta*	Bolivia, Amboró National Park	52
"obobosí"	*Scaptotrigona depilis*	Bolivia, Amboró National Park	53-54
	Melipona sp.	Bolivia, Amboró National Park	55
"suro choco"	*Scaptotrigona sp. aff. xanthotricha*	Bolivia, Amboró National Park	56,57
"suro negro"	*Scaptotrigona polysticta*	Bolivia, Amboró National Park	58,59
"señorita"	*Tetragonisca fiebrigi*	Bolivia, Amboró National Park	60,61
"obobosí"	*Scaptotrigona depilis*	Bolivia, Amboró National Park	62
"abejita"	*Plebeia* sp.	Bolivia, Amboró National Park	63

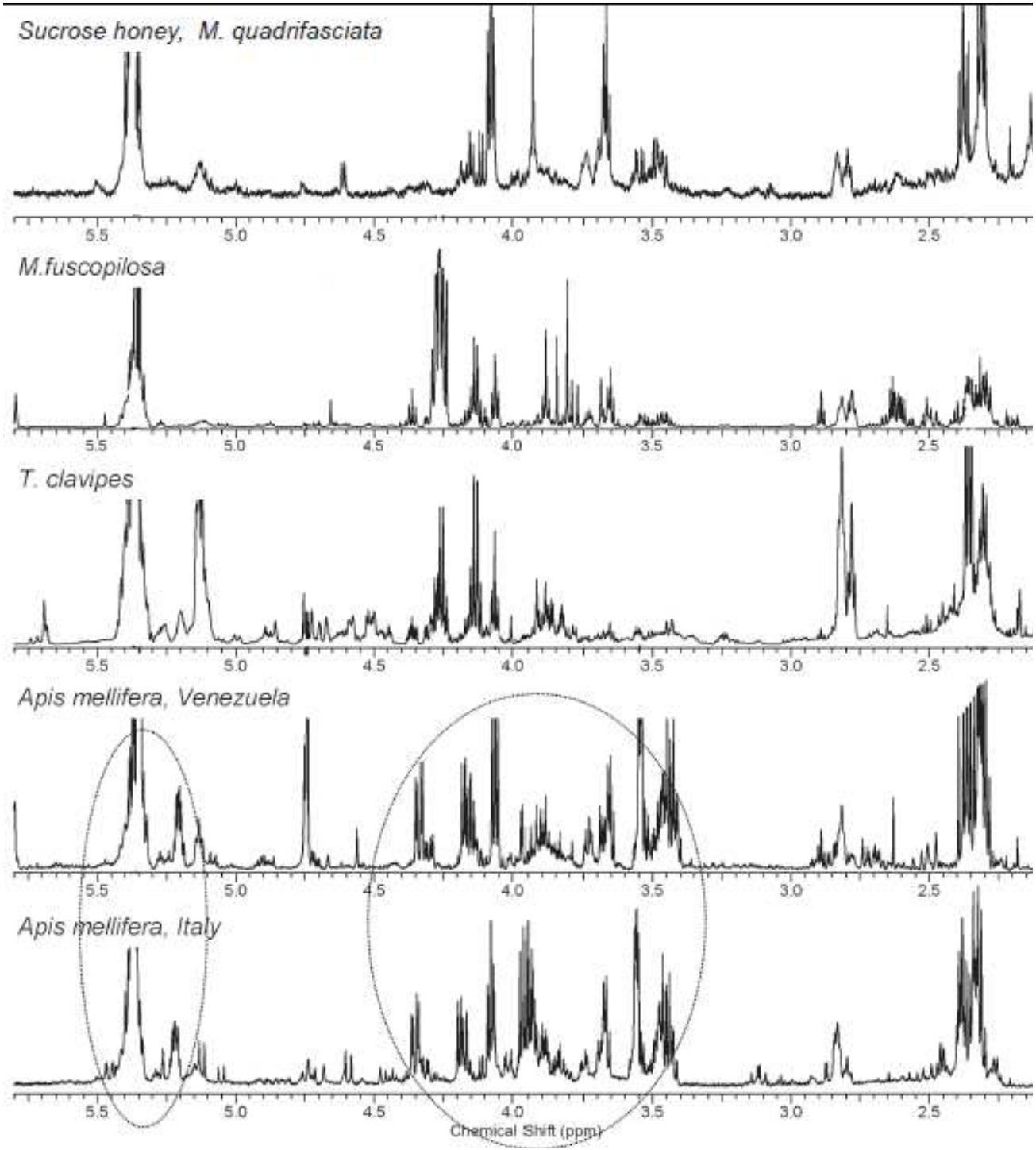








E. Schievano, S. Mammi, I. Menegazzo
In "Pot Honey: A Legacy of Stingless Bees", 2013,
ISBN 978-1-4614-4959-1, DOI 10.1007/978-1-4614-4960-7,
pp. 429-446.


Sucrose honey, M. quadrifasciata
M.fuscopilosa
T. clavipes
Apis mellifera, Venezuela
Apis mellifera, Italy
5.5
5.0
4.5
4.0
3.5
3.0
2.5
Chemical Shift (ppm)

Conclusioni

- Differenziazione botanica
- Dipendenza dall’annata trascurabile
- Marcatori per sei specie diverse
- Differenziazione geografica/entomologica

FEASR

REGIONE DEL VENETO

Fondo europeo agricolo per lo sviluppo rurale: l'Europa investe nelle zone rurali

E. Schievano

C. Facchin

E. Morelato

E. Ndoum

I. Menegazzo

UNIVERSITÀ DEGLI STUDI DI PADOVA

DiSC

M. Stocchero, S.IN Soluzioni Informatiche

M. Panozzo, Rigoni di Asiago